Electroporation of pWTC1 (dd sample) into Rat-1, 1302 and 1-19 cells

5µl of pWTC1 (#4 OVERLAY) was electroporated to each cell line.

	T.C.	Incubation time
Rat-1	21.1	5:00 pm
1302-	17.0	5:45 "
1-19	16.9	6:40 "

RNA extracted according to protocol.

Cell No. for 1-19 cells was very low after electroporation, thus, needs to be repeated.

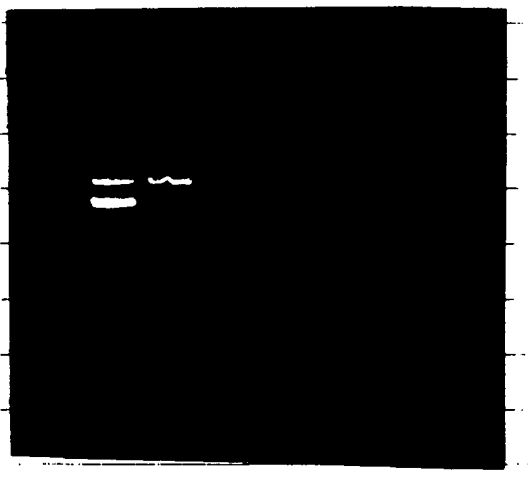
At 2nd isopropanol ppt^n now

1-19 cell electroporation was repeated and samples replated.

Digestion of probe pSTBB0.7 with EcoR1 and gel analysis

5µl of pSTBB0.7 was digested with EcoR1 for 3hr in 50µl RX mix.

An aliquot of digested sample was analyzed on 0.6% agarose gel with undigested vector and the newly prepared pWTC1.



They:

Lane

1 - pSTBB0.7

2 - pSTB0.7 ↓ EcoR1

3 & 4 - pWTC1

EcoRI Digest was heated to 65°C then ppt with 2.2µl 5M NaCl + 120µl ethanol -20° washed with 80% ethanol redissolved in TE at 1µg/µl

RNA purification continued from page 179

20% (40 µl) of each RNA s/n was coprecipitated with 20 µg tRNA and 5µl 3M NaAc pH5.2 + 120 µl ethanol, at −20°, ON.

Rest of RNA samples were stored in presence of 50µl of ethanol at −70°C.

Probe was synthesized according to previous protocol, but since my solutions were used up I used Boehringer* solutions as follows:

µl		Actual
20.5	H_2O	
10.0	5x Buffer *	
6.0	10mM rNTPs (3) *	
1.0	0.1mM UTP *	
2.5	0.1M DTT *	
1.0	RNA guard	
3.0	Template	2µl (2µg)
5.0	^{32}P UTP	7µl 3000 Ci/mmol
1.0	T7 polymerase	1.5µl
50µl		

Final conc^n of cold UTP is 2µM instead of 12µM in my previous experiment, and, of course, the molar quantity of hot UTP is 4-fold lower. Therefore, in the present experiment must do at least 10-fold UTP used.

RNA probe was dissolved in 1ml of hybridization buffer

2µl was used for counting = 162417

ie. total CPM of probe = 500 × 162417 = 8.1×10^7

Normally we use 1.6×10^7 CPM / ml (5×10^5 CPM / 3-pl) for hybridization. In the present situation it is safe to use 3x more, thus using 1 part cond probe to 2 part hybridization sol^n will give 2.7×10^7 CPM / ml 1:1 is about 4×10^7 → used.

For 7 samples used $15 \times 7 = 105$ pl probe
+ 105 pl hybrid^n sol^n

~ 5 pg tRNA used for control
Denatured at 85° / 5 min hybridized 45° o/N

normally we used 1.6×10^7 cpm/ml for hybridization
5×10^5 / 30 µl

It is safe to use 3x more cpm than usual
∴ dilute the concd probe by 1:3

Sequencing gel with wedged spacers used 150 ml 1 acryl sol"

RNA samples (20% of original) were dissolved in 12 µl to
Loading Buffer, 4 µl (1/3) was used for gel loading.

1- Supplement nucleotide mixes with about 2X more CTP cold.
2- Cut DNA template by 1/2
3- add 3X more DNA marker.

Round II RNase Protection pWTC1

RNA samples precipitated with 10 µl of tRNA (100 µg) + 22 µl 3M NaAc pH5.2 at -20°C / 30 min.
Spin at 12000 rpm 4°C. Redissolved pellet in 160 µl DEPC H2O
40 µl (20%) used for RNase protection, ppt'd with 5 µl of 3M NaAc pH5.2 + 120 µl of ethanol, -20 30 min. Spin 5 min 4°C.
redissolve each pellet in 30 µl hybridization buffer + probe.

1 sol'n

µl 60

rNTP stock was made 250 µM in GTP
2 µl of 10 mM sol'n + 2 µl H2O, mix, added 1.5 µl to ~ 37 µl of old stock sol'n. (rNTP - GTP).

2 / 1000 µl of purified probe tested = 240298 CPM

$$\text{total activity} = \frac{1000}{2} \times 2.4 \times 10^5 = 1.2 \times 10^8$$

Used 10 µl of this probe in hybridization sol'n + 20 µl of hybridization solution per sample, O/N incubation 45°C

RNase 1, 30 U (3 µl), 45 min 37°C.

Wedgett gel small teeth used.
RNA samples dissolved in 12 µl, 4 µl (1/3) used for gel loading, ~ 10% of Rat-1 + pWTC1 lost on loading.
Probe: 1 µl added to 30 µl of loading buffer mixed, about 1.5 or 3 µl loaded on gel. DNA markers diluted to about 200 CPM / sample. Gel run at 1350 V

Loading order

PBR322 TaqI DNA | tRNA | (-) Rat-1 | (+) Rat-1 | (-) 1302 | (+) 1302 | (-) 1-19 | (+) 1-19 | Probe | DNA Probe

Xylene Cyanol FF comigrates with a RNA of about 110 nucleotides. Bromophenol blue with 25 nucleotides.

RNA has a lower mobility in these gels than DNA of the same ~~sign~~ length. If DNA markers are used to estimate the size of protected RNA fragment, the correct size is 5 to 10% smaller than this estimate. For example if an RNA species runs with a DNA marker of 100 nucleotides, its actual length is 90 to 95 nucleotides.

Electroporation with pwTC1 (new) 20 µg/Elect.n

Rat-1 340 V Cap 960 µF T.C. = 17.6

Incub.n at 11:40

1302 T.C. 16.9 incub.n started at 12:35

Both cell lines were split the day before electroporation. trypsinization for 2 min.

After electroporation, cells were mixed with 15 ml of medium with a 8 ml of medium, split to two plates, one plate to be used for DNA quantitation, the other for RNA (approx. same no. of cells/plate).

DNA (nuclei) isolated 23 h after and RNA 24 h and 45 min after electroporation

1-19 Cells - electroporation began at 1:45 pm

T.C. = 17.3

~~Cells were counted~~

RNA was purified according to standard protocol, finally dissolved in SDPC treated H2O 65°/10 min. Further purification of RNA samples were also carried out according to protocol. 20 µl of each RNA sample was used for microarray experiments.

New probe was synthesized with the remainder of DNA.

Probe Specific activity:

2.1×10^8 CPM total

Dissolved in 1 ml of hybridization buffer

10 µl (2.1×10^6) of this probe + 20 µl of hybridization solution added to each RNA sample

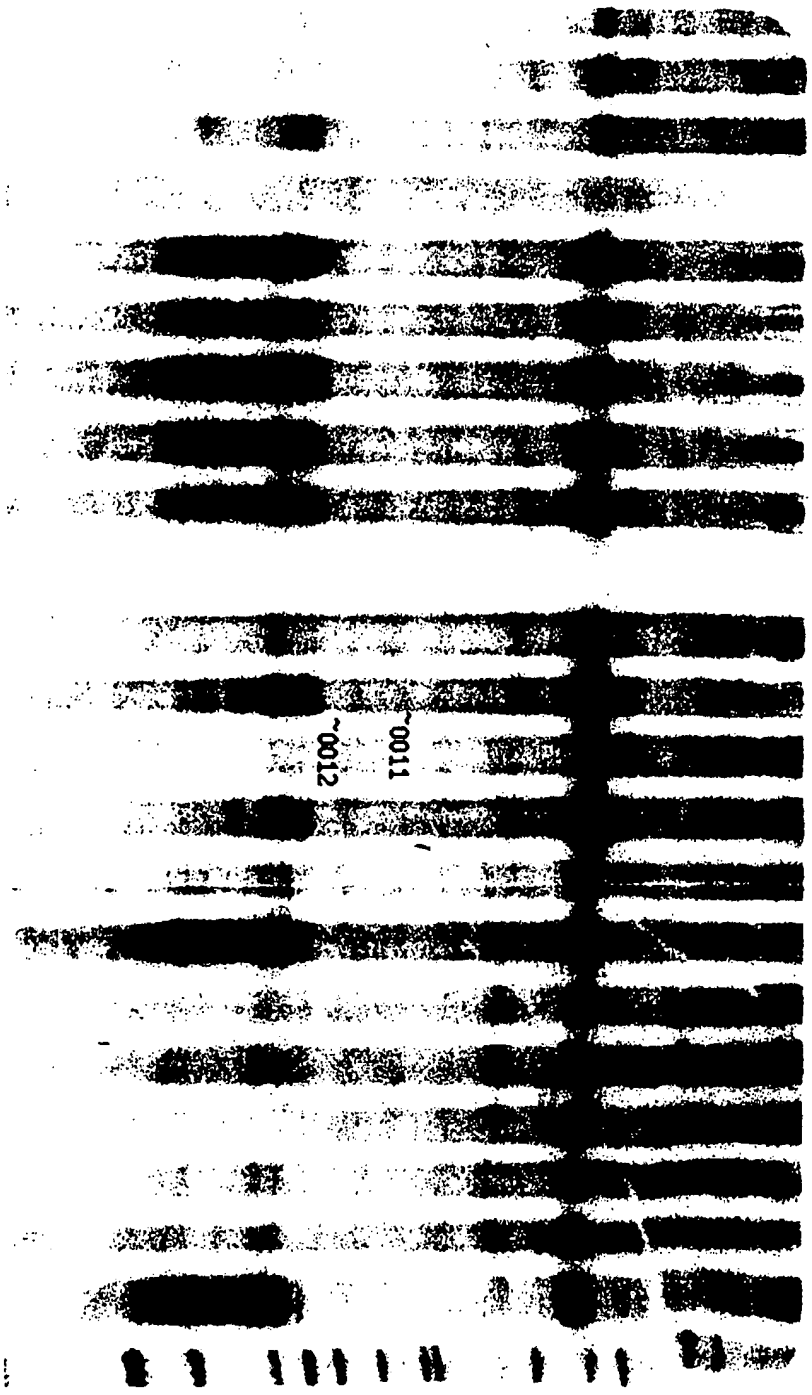
Heated accidentally 7 min (instead of 5 min) at 85°, then incubated at 45°C for 16 h.

2 µl (20 U) of RNase One added / sample digestion was for ~ 75 min.

Samples were finally resuspended in 15 µl of loading buffer, 5 µl was used for gel analysis.

2 vol of 1-19 cells loaded on gel to match the cell numbers for all three cell lines.

∴ 2X DNA must be used



Results of this experiment confirm the previous experiments results that:

1- The steady state level of endogenous Col α1(I) mRNA in 1302 cells is at least 10 fold lower than in Rat-1 cells (compare C2 with C4). The steady state of the mRNA for 1-17 cells is intermediate between the two cell lines, i.e. about 3x higher than 1302 and 3x lower than Rat-1 cells.

2- Electroporation and/or trypsinization causes almost total degradation of endogenous α1(I) mRNA and the level does not return to normality upto 24h after electroporation (less than 10%). This observation is true for all three cell lines.

3- The ectopic α1(I) gene does not seem to transcribe any more upto 24h, i.e. it must be regulated the same way as the endogenous gene

4- The ~~final~~ Col α1(I) truncated promoter constructs synthesize mRNA efficiently within the 24h post electroporation period. For example the human Col-hGH constructs with 820 bp 5' region, or the smaller mouse or rat Col-CAT constructs. Thus the phenomenon of lack of Col α1(I) promoter expression within 24h is only relevant to the fully regulated gene.

199

Elettroporazione di LD/milk pH 8.1 48h incubazione

10 subconfluent plates

20 µg DMT (10 µg F1 + 10 µg F2)

T.C. 15.0

start electroporation at 2:08 (incubation)

CT-3 cont.

positive control

DMT

200

Electroporation of Rat-1, 1302 and BMS-19 with pWTC1 (20 µg) (10µg F1 + 10µg F8 Cs cl purified) (48h)

Control cells: same time all lines trypsinized each passage to harvesting with no electroporation, to differentiate between the effect of electroporation and trypsinization on Cell titer Root degradation and synthesis.

Rat-1	T.C. = 15.9	Incubation began at: 3:58
1302	15.5	4:50
BMS-19	15.8	5:50

Multiple cell Root preparations were done according to standard protocol.
Approximately equal no of cells recovered on each plate after electroporation.

Digestion of probe pST BB 0.7 with Ec[redacted]

pST BB 0.7 DNA 1.45 µg/µl	5 µl	(~ 7.5 µg)
~~10x Root Buffer 3~~ ~~BRL~~ NBB	5 µl	
BamH1 ~~8 units~~ 20 u/µl	3 µl	60 u
H2O	37 µl	

37°C 3h → 65°C 10 min

ppt'd with 2.2 µl 5M NaCl + 120 µl EtOH -20°C washed with 80% [redacted] resuspended in TE [redacted] µl

h)

γ-^{32}P ATP Phosphorylation of pBR322 / MSP I

Fresh NEB DNA Sample	1 µl (1 µg)
H_2O (no heating)	2 µl
10x Kinase buffer NEB	3 µl
γ ^{32}P ATP Fresh	5 µl
T4 polynucleotide kinase 10u	1 µl
	3 µl

3.88

1.50

50 min at 37°C

After spin column purification, 46% of label was incorporated. 60% was left on the column.

Probe was synthesized according to protocol. After column purification was resuspended in 1 ml of hybridization buffer. 10 µl of this solution + 20 µl of cold hybridization buffer added to each RNA sample (1/5 of each RNA sample used).

3 µl of probe solution was taken for scintillation counting which gave 1.3×10^4 counts

Thus CPM / Sample is $13 \times 10^5 \div 3$ µl.

Results of this experiment were almost identical to 24h pate electroporation, i.e. α(1) Collagen mRNA synthesis has not resumed up to 48h.

PCR :

		X 12
10 X Buffer	10 µl	120 ✓
Primer Lac1 / Lac2 Mix	20	240 ✓
dNTP Mix	1.4	16.8 ✓
α^{32}P dCTP (Sweetsold)	3	36
DNATAq polymerase	0.4	4.8
H2O	55	660 ✓
	89.8	
DNA	10 µl	

File #32 10 cycles 1", 94°; 2", 53°; 2', 70°

DNA standards pBAG 50, 10, 5, 1 and 0.1 ng

(Lac primers did not work on pWTC1 Templates, probably because Lac gene is deleted. (insert cloned in pBR322)
New Primers, Amp5 and Amp3 were ordered.

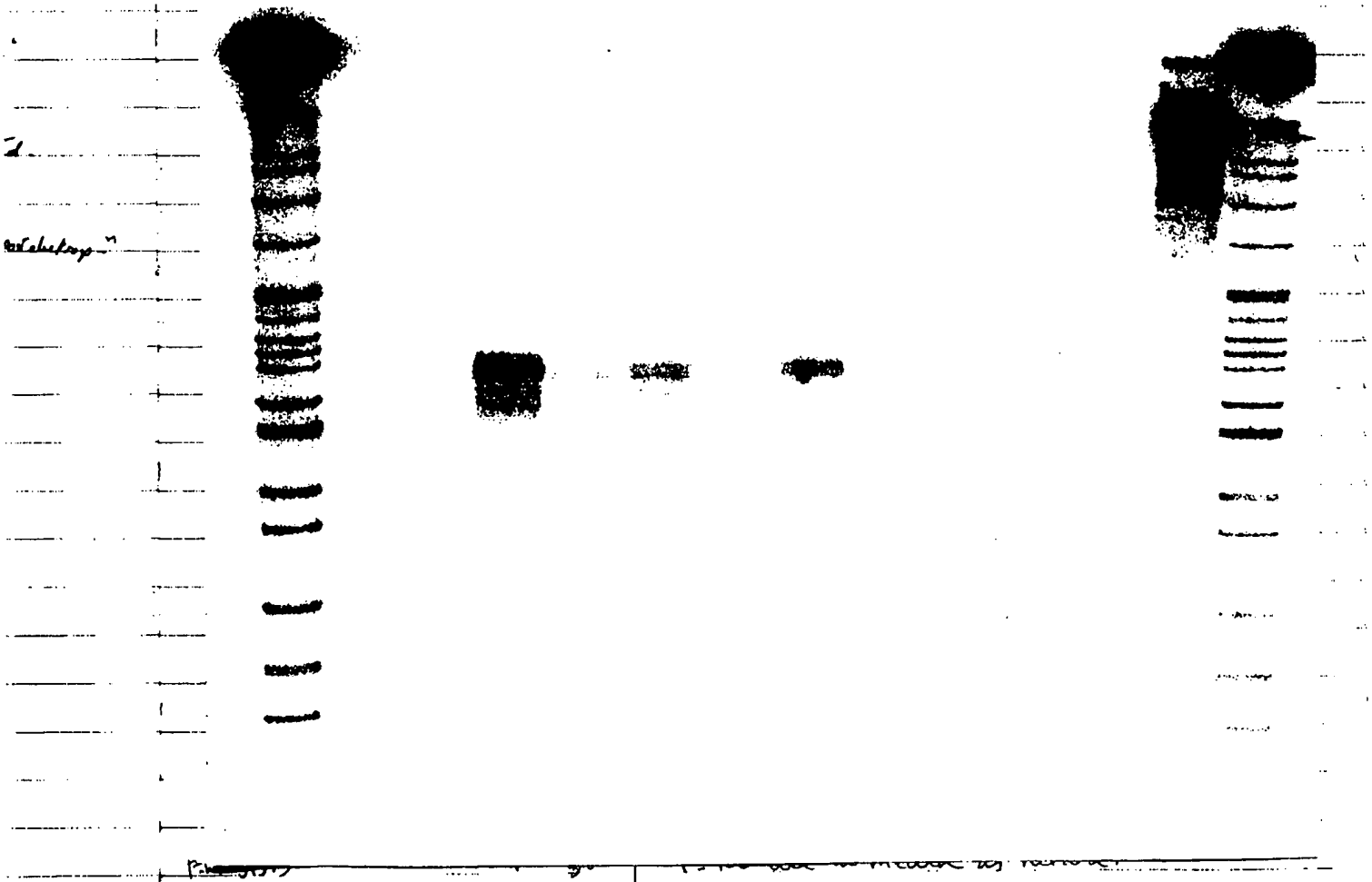
Lanes 1-5: pBAG ; 6: Genomic DNA; 7-12: Rd1, 1302 at 7-19 cells 24 and 48h post electroporation

[redacted]

3-1870

202

A\PWTC48.GEL 12:26:25, Range = 9.98-199.88 Counts, 1.00x



~~The three~~ Transfection of pWTC1 into Rat1 /1302 and 1-19 Cells by DEAE-DEXTRAN - Extended protocol

This was done with 10 µg of DNA in each case according to protocol on page 203.

Cell removal in all three cell lines were very poor, less than 5%. An extra ~~Transfection~~ of 1-19 cells was done without DNA. The yield of cells was just as poor.

Went through with RNA and DNA prep after 48h incubations. 10 µg of tRNA was added to each sample when Guanidine Thiocyanate was added, to help carry the RNA through the procedure.

Intend to run 1/2 of each RNA + DNA sample for each determination. → assuming about equal no. cells were recovered per plate.

These RNA samples will be analyzed along with Uncleotroporated Rat-1, 1302 & 1-19 RNA using another probe such as GAPDH as internal control.

Amp Primers for estimation of Transfected pWTC-1

The cosmid pWTC-1 carries the Amp gene
Two primers were ordered to amplify a 223 b.p. fragment of Amp^r gene. They are designated Amp-5 and Amp-3 Sequences are attached.

Amp-5 was delivered at 13.1 A_{260} 48 µg / A_{260} U
= 628.8 µg
65 µg in 100 µl → 100 µM soln
628.8 µg in $\frac{100 \times 628.8}{65}$
= 967 µl 100 µM soln

Amp-3 11.1 A_{260} at 47 µg / A_{260}
= 521.7 µg
in $\frac{100 \times 521.7}{65}$ = 803 µl = 100 µM

Mix Amp 5 with Amp 3 at equal vol to give 50 µM solution of 2 oligos.
Can use 2 – 4 µl / reaction.

Testing the Amp Primers

The amp primers Amp-5 and Amp-3 mentioned were tested in Colcd PCR Assays as follows:

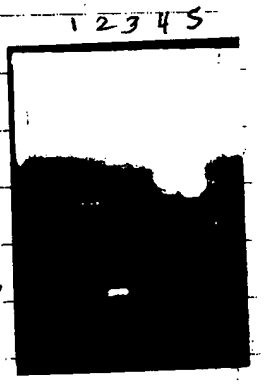
#1) 1 µl of F3 amp tube pWTC1 DNA

#2) 5 µl of 10 ng/µl pBAC Standard (We don't know if pBAC has the Amp^r gene)

	1 sample	2 sample
10 X Buffer	10	20 µl
Amp primers	4	8 µl
dNTP	1.5	3
taq polymerase	0.5	1
H$_2$O	82	164

File #32 25 cycles

After PCR the products (10µl each) were analyzed on 1% agarose gel along with ØX174 HaeIII markers and two plasmids received from Benoit: pGroCT and pLS-1, for quantitation. 1/2 µl of the two plasmids loaded on gel.



Thus:

Lane 1 = ØX174/HaeIII

Lane 2 = pWTC1 PCR product → pure plasmid

3 = pBAC " " + rat genomic DNA

4 = pGroCT

5 = pLS-1

Conclusions: Primers work well on both pWTC1 and pBAC templates. At least two additional bands apparent - the gel came from genomic DNA.

pGroCT conc'n is ~1 µg/µl pLS1 ≈ 3-4 µg/µl. ✓

Further Studies on the Fate of Endogenous Collagen α1(I) mRNA after electroporation

There are a number of questions to be asked regarding the mechanism of Collagen α1(I) mRNA degradation and cessation of synthesis:

1- Is it electroporation per se responsible of the shut off or electroporation + DNA?

2- Is it the cosmid with the complete α1(I) gene which is titrating certain transcription factor(s) and resulting with lack of endogenous mRNA?

3- Does it synthesis of α1(I) mRNA resume after several days, or the process is irreversible?

In order to answer the first question an electroporation of KD-1 cells was done without DNA, incubation was continues for under 48 h initially, some of the cells were removed for mRNA analysis, the rest replated and continued incubation for an extended time to take care of questions 1 and the 3.

The second question is already partly answered: electroporation with cosmid does not result in cosmid expression, neither the cosmid nor the endogenous gene are expressed. Moreover electroporation is never 100% efficient, thus it should not result in 90-95% drop in the level of endogenous gene expression. In any case, an electroporation was done with 3 μg of pCol CAT-3 which carries the basal α1(I) promoter construct. It is electroporated very efficiently, we also know the it expresses CAT gene efficiently, we shall investigate if it with Turn off endogenous α1(I) mRNA

Cells were harvested, for CAT-3 / Rat-1 at 45 h, plate was 80% confluent, compared to cosmid electr. which was about 60% confl. 1/2 cells to RNA prep, the other 1/2 to DNA.

Electroporated cells without plasmid were 100% confluent at 48h. 40% of the cells were used for Rat prep, the rest of the cells were split-ran diff plates for further incubation and study.

So about 1/5 of each Rat sample should be equivalent to previously electroporated Rats, but again internal control pools need to be used to be able to compare results.

Electroporated cells harvested again at 5 days + 7 days post-electroporation. At 5 days a 30% confluent plate used, at 7 days plate was 90% confluent. Thus Rat conc's need to be determined before protection experiments.

PCR of all DNA samples with New AMP-primers

	1x	17x
10 x Buffer	10 µl	170 ✓
Primers Amp 3 / 5	4	68 ✓
dNTP Mix	1.4	24 ✓
α 32p dCTP	1	17
Taq polymerase	0.4	7
H2O	73	1241 ✓
	90 µl	
DNA	10 µl	

File #32 / 33 12 Cycles.

Lane 1 = Rat-1 / pWTC1, 24h post electroporation
2 = 1302 " " " "
3 = 1-19 " " " "
4 = Rat-1 / pWTC1, 48h post electroporation
5 = 1302 " " " "
6 = 1-19 " " " "
7 = Rat-/pWTC1 48h post DEAE-DEX Transfection
8 = 1302 " " " "
9 = 1-19 " " " "
10 = Rat-1 Genomic DNA
11 = Genomic DNA + pBAG 0.1 ng / 10µl
12 = " " " " 1 " "
13 = " " " " 5 " "
14 = " " " " 10 " "
15 = " " " " 50 " "
16 = Rat-1 / pCDM7-3, 45 h post electroporation - 10% DNA used

10% of DNA used

50% of DNA used.

* 10% of all PCR reactions analyzed on gel.

Results show that all transfection experiments worked equally well. Controls did not work, presumably pBS14 does not have the Bmp gene. Control genomic DNA cross contaminated with adjacent samples.

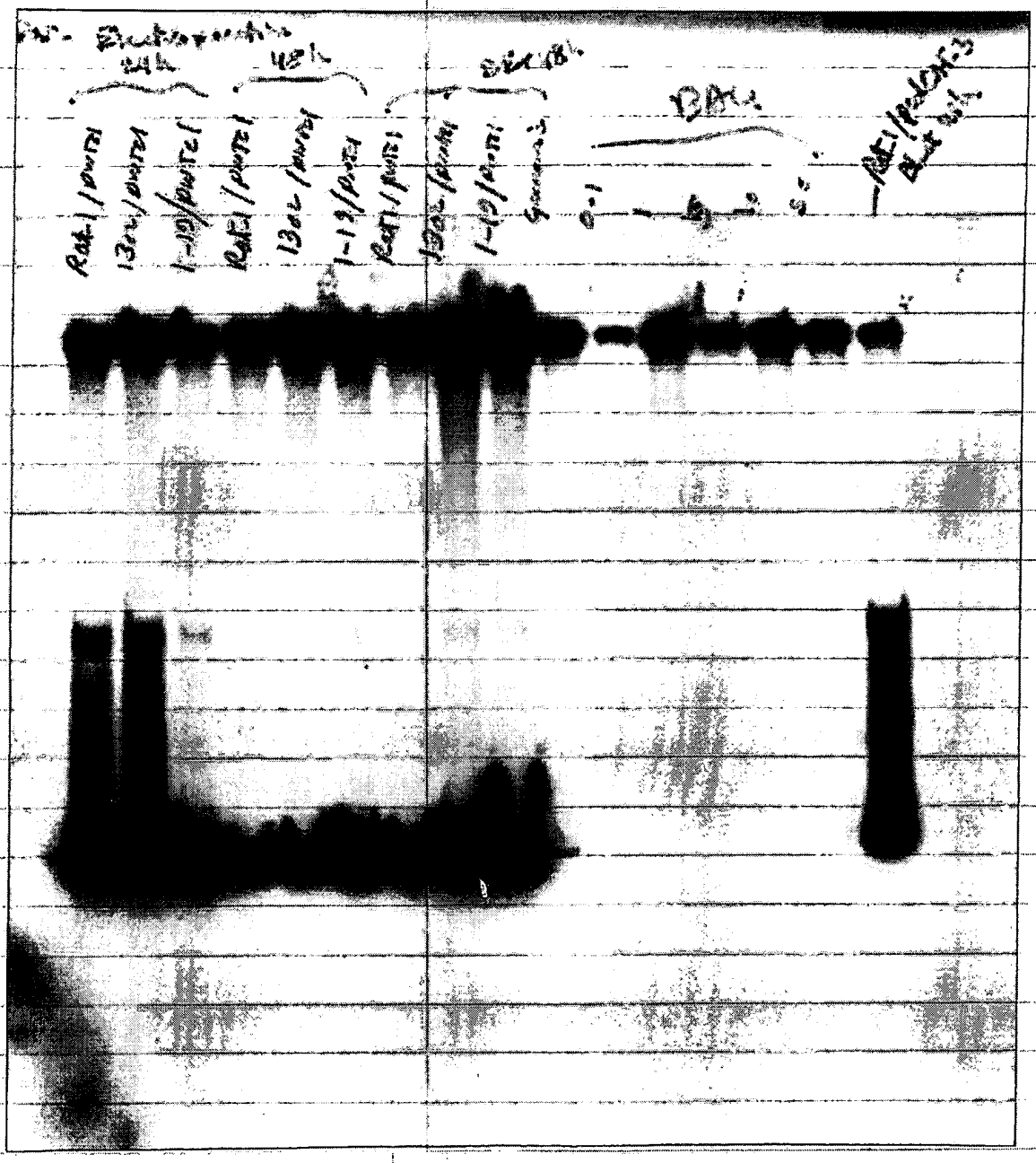
PCR was repeated for the new controls under exactly the sample conditions as the last experiment. The new standards are made from pCol CAT-3. Concentrations are: 1, 10, 50 and 100 ng / 10µl. negative control, genomic DNA was also repeated. 5µl of each reactions loaded on the gel.

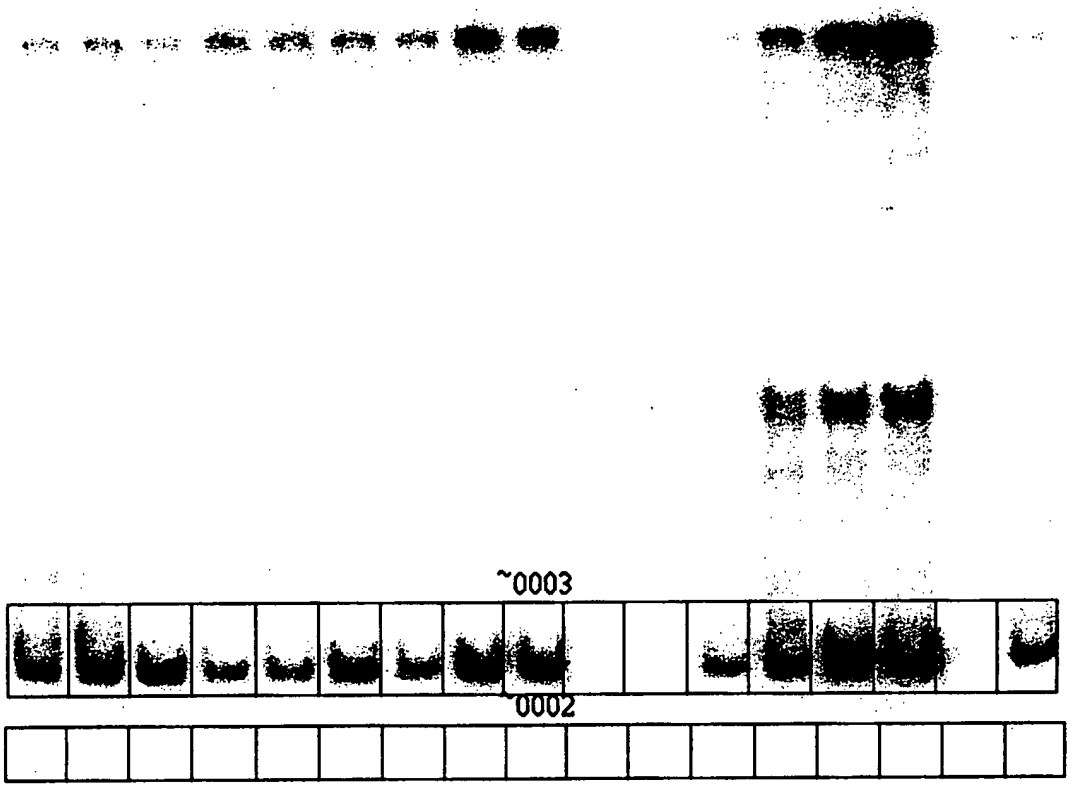
1 space allowed on gel between samples 9 and 10, and between 100 ng standard and pCol CAT 3 / RAT1 DEX. Gel ran at 200 V

5µl of DPB dye + 5µl sample -

Gel No 2: about 20% of the sample in Lane #2 was accidentally lost - floated.

The second gel worked fine all of the standards showed up with A/N expression and Genomic DNA did not.

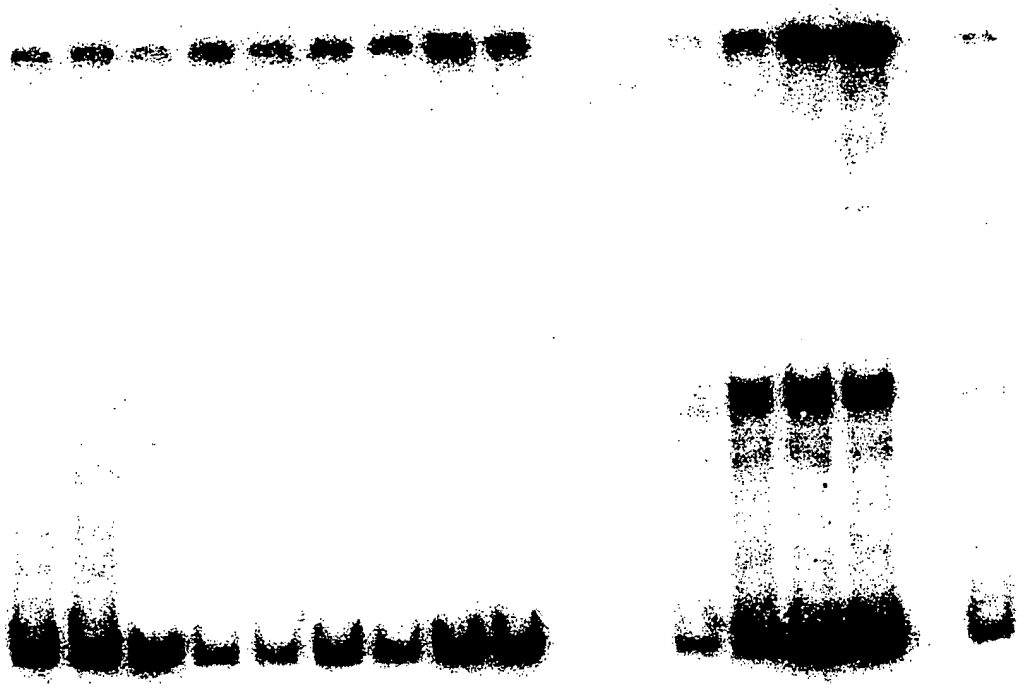


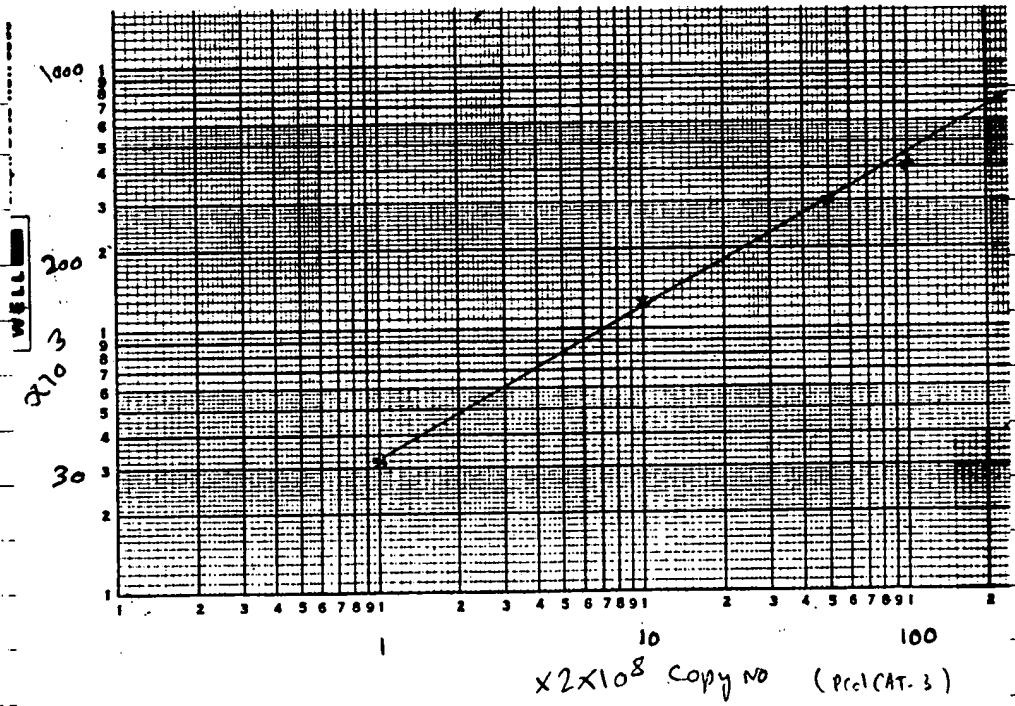


OBJECT NAME	VOLUME	ng	BACKGROUND	# PTS IN OBJECT	SUM ABOVE BKGND
~0003-R1C1	135099	12	11.65	3243	136823
~0003-R1C2	133605	12	14.18	3243	135889
~0003-R1C3	133183	12	11.06	3243	138977
~0003-R1C4	29565	0.92	8.836	3312	33412
~0003-R1C5	32578	1	8.423	3243	35498
~0003-R1C6	77621	4.6	8.065	3243	81280
~0003-R1C7	40320	1.4	9.031	3312	45322
~0003-R1C8	142898	13	14.03	3243	150019
~0003-R1C9	124376	10.5	19.63	3243	138473
~0003-R1C10	-15962		11.63	3243	4899
~0003-R1C11	-378.2		3.427	3312	1288
~0003-R1C12	32414		7.658	3243	34399
~0003-R1C13	123946		17.12	3243	128648
~0003-R1C14	305291		27.42	3312	315661
~0003-R1C15	405947		30.79	3243	417083
~0003-R1C16	-18125		15.00	3243	6108
~0003-R1C17	50548	2.2	6.263	3243	58442

Amp gene Copy #
24×10^9
30×10^9
24×10^9
1.84×10^9
2×10^9
9.2×10^9
0.56×10^9
5.2×10^9
4.2×10^9
4.4×10^9

Gene Copy# = ng (read from graph) $\times 2 \times 10^8 \times 1$ / Fraction of DNA used in PCR





Amp
Copy #
8.4 x 10^9
3.0 x 10^9
2.4 x 10^9
1.84 x 10^9
2 x 10^9
9.2 x 10^9
0.56 x 10^9
5.2 x 10^9
4.2 x 10^9

4.4 x 10^9

XR

Digestion of probes pLS-1 and pGract with Hind III

pLS-1 (1 μg/μl)	10 μl
BRL 10X React Buffer 2	5 μl
Hind III 20u/μl	2 μl
H2O	33
	50 μl
pGract 4 μg/μl	2.5 μl
BRL 10X React Buffer 2	5 μl
Hind III 20u/μl	2 μl
H2O	40 μl
	50 μl

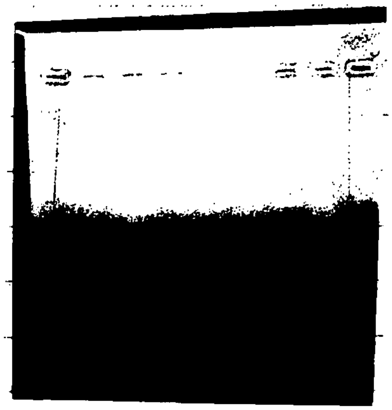
37°C / O/N

Analysis of RNA

RNA Samples from Rat1 clut.pos... after 2, 5 and 7 days and CAT-3 / Rat-1 clut. 48h, and unclut... Rat-1 (48h) were quantitated. 5% of each Rat sample ~~(... μl) and ... for 7 Days 2.5% were mixed with RNA blue dye ... dyes and analyzed on polyacrylamide gel~~

Analyzed on 1.5% agarose gel along with HindIII digested probes. (1 μl of 20 μl soln in TE)

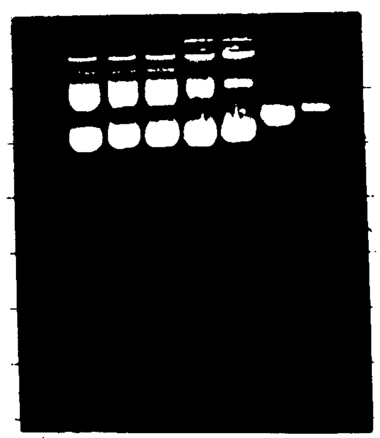
Lane 1: pGract ↓HindIII
2: pLS-1 ↓HindIII



Lane
4% 1): Unelectroporated Rat-1 RNA (48h)
16% 2): Elect^d Rat-1 RNA, 45h
30% 3): " " " 5 days
90% 2.5% used ← 4): " " " 7 days
40% 5): Rat-1 electroporated with pCiCAT-3, 45h
6) pLS-1 / HindIII ↓
7) pGract / HindIII ↓

pLS-1 seems at least 2X stronger than pGract; ∴ pLS-1 was diluted 2X so that both probes will be about 0.5 μg/μl

The RNA concentrations do not seem to correspond to visual cell no. Could also be artifact since electrophoresis was done in TBE pH 8.2 in which RNA may not be so stable.



Experiment was ∴ repeated.

	Cell/ Electroporation	Visual confluency	Actual cell No. Coulter counter	Resuspend RNA in X µl TE
Summary				
	Unelectroporated	60-70%	1,840,000	184
	Elect'd — 2 Days	90%	3,200,000	320
	" 4 Days	80%	2,040,000	204
	" 6 Days	70%	2,080,000	208
Electroporated Rat/CAT-3 — 2 Day		80% → 1/2 to RNA, ∴ ~ 40%		102

Use 20 µl of each RNA sol'n / experiment
+ 2 µl tRNA
+ 2.5 µl 3M NaAcetate pH 5.2
+ 75 µl ethanol
⇒ — 20°C 30 min Spin 4°C / 5 min

suspend in RTE

Repeat of Electroporations of Rat-1, different cell harvest times, County of cells by Coulter Counter.

Unelectroporated Rat-1 cells look about 60-70% confluent

Electroporated Rat-1 cells after 2 days look about 90% confluent

Cell counts: Elect 2 Day 8000

Unelectroporated 4800 4645

8000 X 40 X 10 = 3,200,000 — 2 D

4600 X X40X10 = 1,840,000 — Unelectroporated

4 Day cells look about 80% confluent:

5100 x 40 x 10 = 2040,000

5 Day cells look ~70% confluent

5200 X 40 X10 = 2,080,000

3 probes, pSTBBa-7, pLS-1 and pGraet were synthesized according to protocol, except incubation was accidently increased by ~20 min (50 min instead of 30)

Incorporation of different probes after purification was similar by Cigen counter ($\sim 3.5 \times 10^5$)

Each probe was diluted in 1 ml of hybridization buffer.

The probes were mixed in the following ratio:

	per sample µl	×24
pSTBBa-7	10	240
pLS-1	5	120
pGraet	3	72
Hybrid. Sol.	12	288
	30 µl	720 µl

22 hybridizations were performed:

(6) original 24h post electroporation } 20% of RNA used
(6) " 48h " "
(4) DEAE-DEXTRAN Transfection 48h incubation. 50% of RNA used
(4) 1 unelectroporated, 2 days, 4 days and 6 days post electroporation } 10% RNA used
(1) Rat-1/CAT-3 elect. 48h
(1) tRNA control.

Sequencing gel:

1/30/ 60 ml 6% acrylamide / 1 x TBE / 7.7 M Urea
400 µl 10% APS
40 µl TEMED.

Lane: 1) pBR322 ↓ Msp I

Exp 1: Electroporation of Rat-1 / B02 / 1-19 with pWTC1 - 24 h post elut"

2) Rat-1, unelectroporated
3) Rat-1 Electroporated
4) 1302, unelectroporated
5) 1302 elutroporated
6) 1-19, unelutroporated
7) 1-19 elutroporated

Exp 2: Electroporation of Rat-1 / 1302 / 1-19 with pWTC1 - 48h post elut"

8) Rat-1 unelectroporated pretrypsinized (P/T)
9) Rat-1 Elutroporated
10) 1302 unelutroporated P/T
11) 1302 Electroporated.
12) 1-19 unelutroporated P/T
13) 1-19 Electroporated

Exp 3: Electroporation of Rat-1 cells with pCdCAT-3 48h

Exp 4: 14) Rat-1 / pCdCAT-3 48h post elutroporation

Exp 4: Unelutroporated Cells VS Electroporated Rat-1 cells, 2 4 and 6 days post elut"

15) Rat-1 unelutroporated
16) Rat-1 Electroporated no plasmid 2 days post elutroporation
17) " " " 4 " "
18) " " " 6 " "
19) τ RNA Control

Expt 5; Transfection of pWTC1 into Rat-1, 1302 and 1-19 by DEAE-DEX 48h

20) Rat-1 /pWTC1 DEX 48h

21) 1302 /pWTC1 DEX 48h

22) 1-19 /pWTC1 DEX 48h

23) 1-19 ~~Unelectroporated~~ DEAE-DEX Treated

24) Mixed 3 probes in the ~~cocktail~~ preparations added to hybridization mixture

25) probe pST BBO-7

26) probe pLS-1

27) probe pGrant — negative control for Rsam protection

28) pBR 322 √MSP1

24 h postelectroporation | 48 h

	Rat-1 unelectroporated	Rat1+pWTC1	1302 unelectroporated	1302+pWTC1	1-19 unelectroporated	1-19+pWTC1	Rat-1 unelect. + trypsinized 16h before	Rat-1+pWTC1	1302 trypsd. unelectrop'd	1302+pWTC1	1-19 ...
α1(1) (main bands, 2)	~~48727~~	~~7128~~	~~6007~~	~~1942~~	~~9094~~	~~6184~~	~~85264~~	~~11223~~	~~26820~~	~~5357~~	~~48343~~
- 1/2 tRNA (2800)	45927	4328	3207	[illegible]	6294	3384	82464 120,000	8423	24020	2557	45543
GAPDH	~~45902~~	~~28635~~	~~38668~~	~~31673~~	~~43298~~	~~122744~~					
-1/2 tRNA (2000)	44951	43730	25752	36590	27847	39501	113911	38684	173901	65275	154372
α1(1) / GAPDH	1.023	0.100	0.124	0 UD	0.226	0.086	0.724 1.05	0.218	0.138	0.039	0.29

α1 (background subtraction)

	OBJECT NAME		VOLUME
	~0005-R1C1	.2[illegible]	4780
	~0005-R1C2	.254	8118
	~0005-R1C3	.072	2582
	~0005-R1C4	.608	11417
tRNA	~0005-R1C5	—	2837

GAPDH

	OBJECT NAME	VOLUME
	~0002-R1C1	18677
	~0002-R1C2	12265
	~0002-R1C3	35873
	~0002-R1C4	18776
tRNA	~0002-R1C5	3830

Oligos Etc. P.O. Box: 874 Ridgefield, CT 06877

FAX # 800-869-0813

Phone: 800-888-2358

P. O. No.: GFR-550480

Ship To: Dr. M.B. BAHRAMIAN / Dr. ZARRL

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Room E18-566, 400 MAIN ST, CAMBRIDGE, MA 02139

Phone: (617) 253 7666

BILLING ADDRESS: MIT, P.O. Box 69, CAMBRIDGE, MA 02139

Oligo # AMP-5:

5' gTA gTT CgC CAg TTA ATA gT 3' 20-mer

Small scale

Oligo # AMP-3:

5' gCT gCC ATA ACC ATg AgT gA 3' 20-mer

small scale

Date required by: ASAP

40 nucleotides at $4.50 = $180.00

Feb. 23, 1995

M.B. Bahramian

Amplifies a 223 bp fragment from Ampicillinase gene cloned into pBR327

Soberon, X., Covarrubias, L. and Bolivar, F. (1980) Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene 9, 287-305.

48h post-elec.

48h post transfection

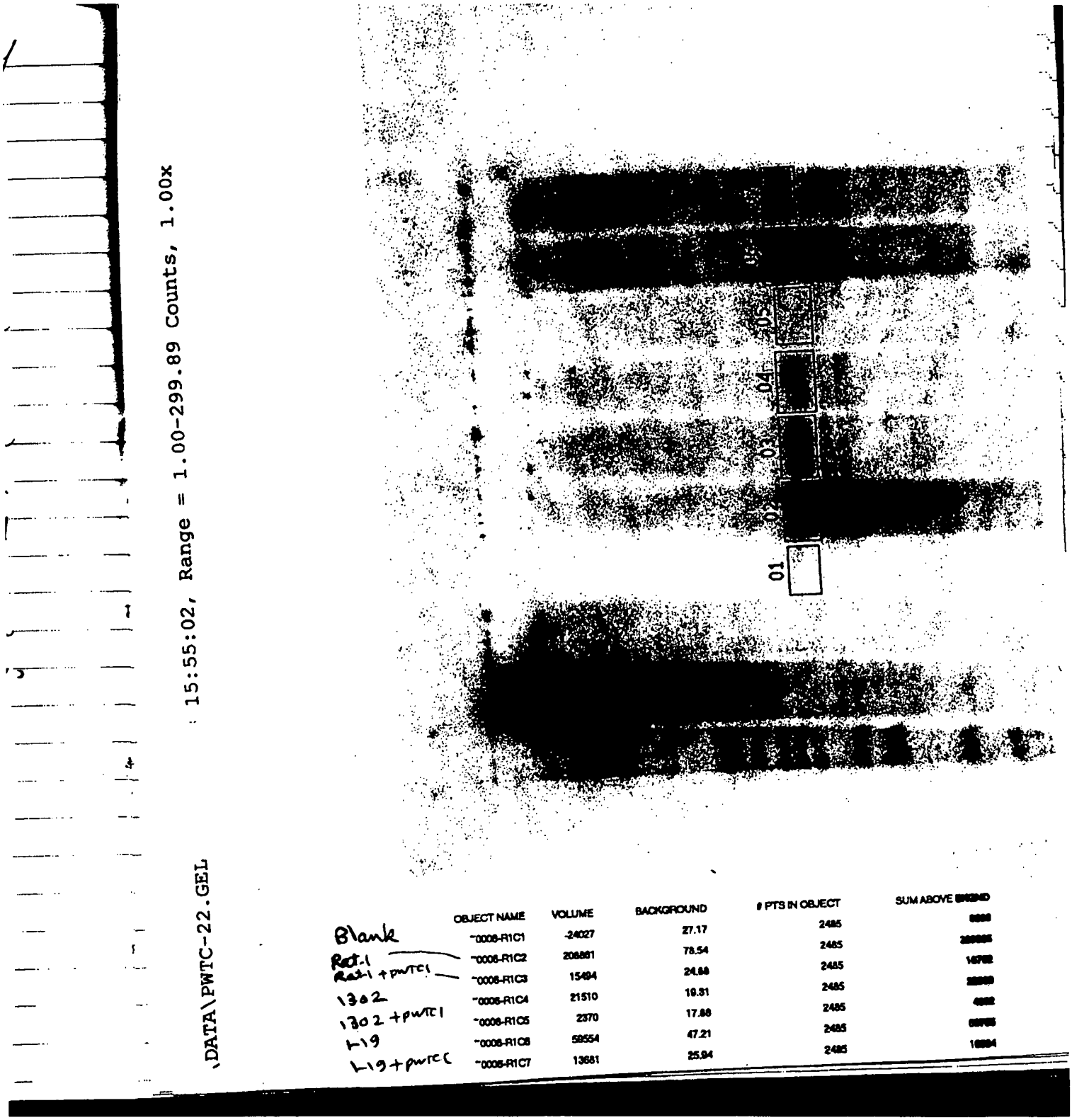
1302 trypd. undestrop d.	1302+pwTcl	1-19 tryp undest.	1-19+pwTcl	Rat-1 pcolCAT-3	Rat-1 undestroporated	Rat-1 2 Days post electroporation	Rat-1 4 Days post electrop	Rat-1 6 Days post electrop	Rat-1+pwTcl DEAE-DEXTRAN	1302+pwTcl DEAE-DEXTRAN	1-19+pwTcl DEAE-DEXTRAN	1-19 DEAE-DEXTRAN
~~6820~~	~~5357~~	~~48343~~	~~11193~~	~~46214~~	~~62539~~	~~63824~~	~~37708~~	~~57216~~	10284	1449	~~1983~~	~~3617~~
									~~7484~~	~~0~~	~~0~~	~~817~~
1020	2557	45543	8393	43414	59750	61024	34908	54416	10017	1182	1716	3350
								~~61330~~	~~14946~~	~~32049~~	~~8495~~	~~14847~~
73801	65275	154372	57075	88930	59500	53751	44382	63163	16776	33873	10265	16677
								0.862	0.597	0.035	0.167	0.201
2.138	0.039	0.295	0.147	0.488	1.004	1.135	0.787	~~0.880~~	~~0.548~~	~~0.045~~	~~0.235~~	~~0.244~~

α1(I)

	OBJECT NAME	VOLUME
tRNA	~0014-R1C1	5585
Rat1 6D	~0014-R1C2	57216
Rat1 4D	~0014-R1C3	37708
Rat1 2D	~0014-R1C4	63824
Rat-1 U	~0014-R1C5	62539
Rat-1/CAT3	~0014-R1C6	46214
—	~0014-R1C7	685.7
1-19/pwTcl	~0014-R1C8	11193
1-19	~0014-R1C9	48343
1302/pwTcl	~0014-R1C10	5357
1302	~0014-R1C11	26820
Rat-1 pwTcl	~0014-R1C12	11223
Rat-1	~0014-R1C13	85264
1-19/pwTcl	~0014-R1C14	6184
1-19	~0014-R1C15	9094
1302/pwTcl	~0014-R1C16	1942
1302	~0014-R1C17-	6907
Rat-1/pwTcl	~0014-R1C18	7126
Rat-1	~0014-R1C19	48727

GAPDH

	OBJECT NAME	VOLUME
tRNA	~0006-R1C1	4089
	~0006-R1C2	65163
	~0006-R1C3	46382
	~0006-R1C4	55751
	~0006-R1C5	61500
	~0006-R1C6	90930
	~0006-R1C7	1734
	~0006-R1C8	59075
	~0006-R1C9	156372
	~0006-R1C10	68275
	~0006-R1C11	175901
	~0006-R1C12	40684
	~0006-R1C13	115911
	~0006-R1C14	41501
	~0006-R1C15	29847
	~0006-R1C16	38590
	~0006-R1C17	27752
	~0006-R1C18	45730
	~0006-R1C19	46951



	OBJECT NAME	VOLUME	BACKGROUND	# PTS IN OBJECT	SUM ABOVE BKGND
Blank	~0006-R1C1	-24027	27.17	2485	[illegible]
Rat-1	~0006-R1C2	206881	78.54	2485	[illegible]
Rat-1 + pwTC1	~0006-R1C3	15494	24.88	2485	16782
1302	~0006-R1C4	21510	19.31	2485	[illegible]
1302 + pwTC1	~0006-R1C5	2370	17.88	2485	4082
L-19	~0006-R1C6	59554	47.21	2485	[illegible]
L-19 + pwTC	~0006-R1C7	13681	25.94	2485	16884

224

Electroporation of Rat-1, 1302, 1-19 Cells with:
CAT-1 (3.6 Kb), CAT-2 (2.5) and CAT PVUII (~1Kb) and CAT-3 (0.22kb):

Determination of transcription and translation in vivo RNA / Cell extract and Nuclei isolations (each electroporation divided 3 ways.

Electroporation of NIH 3T3 Cells with pWTC1

RNA / DNA isolation - determine that rat & mouse cells behave similarly with respect to α1(I) shut off by pWTC1.

Not enough CAT-1 was present, so it was prepared by boiling method. The yield of plasmid was bad, so same old and some new plasmid were mixed and used in various electroporations.

Viability of 1-19 cells after elect^{n} was good 1302 & Rat-1 cells not so good (about 1/4 to 1/5 of 1-19). 3T3 cells recovery was bad. 3T3 cells were re-electroporated same day the second prep was OK., same ~~bad~~ as recovery as 1302 + Rat-1.

Following experiments are planned to eventually run on 4 gels

(1) 4 different size α1(I) promoter – CAT gene constructs transfected into Rat-1, 1302 and 1-19 cells (12 samples)
+ 3 controls Rat-1, 1302, 1-19 untransfected (48 h after electroporation)
+ 1 tRNA lane (16 samples + DNA and probe lanes)
Mix CAT probe + GAPDH probe (only to serve as positive control for hybridization and to show that comparable number of cells were used
DNA det^ by PCR will show the relative rates of transcription.

(2) Exactly the same RNA samples as in (1) used but probed with α1(I) probe and GAPDH probe to investigate the extents of suppression of endogenous rat α1(I) collagen gene by different lengths of the transgenic promoters

(3) Effect of exon-intron region and whole gene pWTC1 on Rat-1, 1302 and NIH 3T3:

Rat-1 RNA Control	Rat-1/pST BB2-6	Rat-1/pST BB0-7	1302 Control	1302/ BB2-6	3T3 cont	3T3/ BB2-6	3T3/ BB0-7	3T3/ pWTC1

+ tRNA lane (10 samples)

Probe with α1(I) (pST BB0-7 ↓ EcoRI antisense) and by GAPDH
Endogenous and exogenous α1(I) protected fragments will comigrate, except in the case of pWTC1 transfected 3T3 cells
The short promoter + exon-intron regions are expected to express α1(I) mRNA at lower levels and should theoretically lead to increase in the total α1(I) mRNA / GAPDH mRNA ratios. If the opposite takes place, then murine/rodent promoters and human α1(I) gene are not regulated by the same factors.

Electroporation of pSTBB 0.7 and pSTBB 2.6 into NIH-3T3, Rat-1 cells and 1302.

The objects of this experiment is to verify if the shorter or longer intronic/exonic sequences have any effect on expression of the endogenous α(1) in both Rat-1 cells and in NIH 3T3 cells. Also want to investigate if the short mouse promoter with the 5' intron/exon sequence (pSTBB2.6) is expressed to similar levels in 1302 cells.

Thus ~~was~~ electroporated Rat-1 & 3T3 cells with 0.7 & 2.6 plasmids at 1302 with just 2.6 plasmid, a control NIH3T3 was also electroporated at the same time with no DNA, for verification of endogenous α(1) mRNA control.

RNA probes to be used are pSTBB 0.7 and GAPDH also DNA will be determined.

We could also use ~~antisense~~ sense pSTBB 0.7 for antisense mRNA synthesis in embryos.

Recovery of cells after electroporation was good for Rat-1 & 1302 cells but not good in NIH-3T3 cells (30%), with 0.4 & 1.6 10%. For Rat-1 almost 90% after 48h.

So for Rat-1 & 1302 ~~was~~ should use 1/5 of flask for 3T3 1/2 of flask.

α1 (I) sense RNA probe pSTBB0.7 was digested with PST-1 (NEB Buffer #3) 4h, heated to 65°C/15min 10μg in 20μl reaction vol, without further purification proceeded to riboprobe synthesis.

PBR322 ↓ MSPI was labelled with γ ^{32}P to about 10⁶ gigen counts / 2μg DNA with about 60% of labels incorporated into DNA on spin column purification. 2μl of 300μl elusate was mixed with 50μl of RNA loading buffer which gave 100,000 gijen counts / 50μl 2,000 counts / μl less than a μl sufficient on gel

3 Probes (pST BB0.7, α1(1); pLS-1, GAPDH; and pTRI-CAT) were transcribed by T7 RNA polymerase according to protocol.

A second point of this investigation is to verify that short promoter + the enhancer elements is ~~to~~ 2-fold less efficiently expressed in 1302 cells compared to Rat-1, in accordance with the data on human procollagen α1(I) - hGH reported results. ∴ DNA data supported

(4) To investigate if significant increase in anti-sense α1(I) mRNA results in shut-off of this gene.

24h post transfection

Rat-1 (Control)	Rat-1/ pNTC1	1302	1302/ pNTC1	1-10	1-10/ pNTC1	3T3	3T3/ pNTC1

Rat-1/ BB0.7	Rat-1/ BB2.6	1302/ BB2.6	3T3/ 0.7	3T3/ 2.6	tRNA

Probe with sense α1(I), BB0.7 ↓PST-1 + GAPDH

4 probes to be synthesized: BB0.7 sense + anti-sense GAPDH and CAT

1/3 of the above RNA samples to be used / experiment

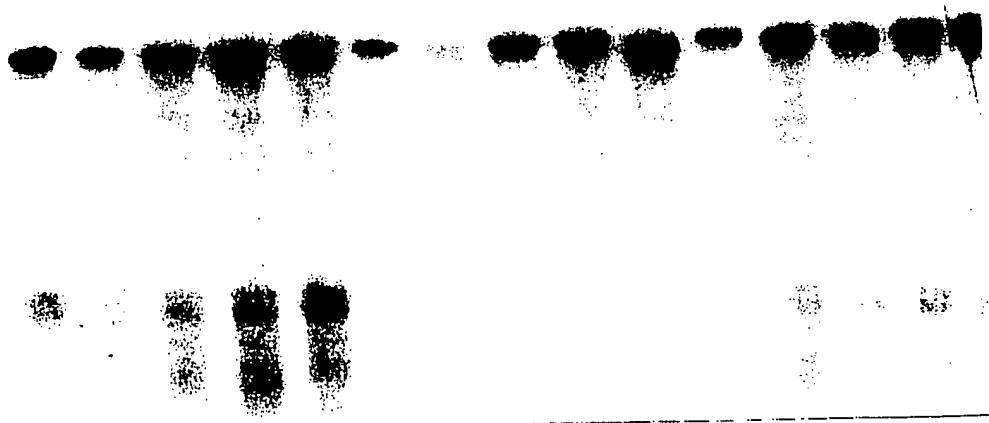
except for 24h elut: Rat-1 1302 1-10 as I have run the remaining RNAs

Results

1st gel (CAT / GAPDH) came out black in all lanes including RNA presumably because RNA contained some NaOH which might inhibit RNase one. Thus this experiment should be repeated with 1/2 of RNA samples left, with new probes.

Gel #2 came out ok. (αV(1) / GAPDH)

	OBJECT NAME	αV(1) VOLUME	GAPDH VOLUME	αV(1)/GAPDH	
tRNA Rat-1	"0013-R1C1	10552	1888	1.56	1.02
Rat-1 3.6 Kb	"0013-R1C2	84059	53787	0.74	0.47
" 2.5	"0013-R1C3	53236	71792	0.68	0.44
" 1.0	"0013-R1C4	131513	193460	0.75	0.48
" 0.22	"0013-R1C5	212066	283413	0.77	0.49
1302	"0013-R1C6	182131	235120	0.45	0.16
1302 /3.6Kb	"0013-R1C7	22918	50539	0.27	0.05
" 2.5	"0013-R1C8	5051	18814	0.18	0.05
1.0	"0013-R1C9	18825	104257	0.18	0.06
0.22	"0013-R1C10	31147	174568	0.14	0.05
1-19	"0013-R1C11	28895	206870	0.49	0.25
1-19 /3.6	"0013-R1C12	24611	50129	0.52	0.26
2.5	"0013-R1C13	49208	94022	0.39	0.20
1.0	"0013-R1C14	39977	100697	0.35	0.18
0.22	"0013-R1C15	45788	128228	0.43	0.22
	"0013-R1C16	39801	91761		



Gel #3 : 1/2 of the samples related to Rat cells came out ok. but mouse cells did not work since Mouse and rat GAPDH are different and specific mouse probe must be used

	OBJECT NAME	VOLUME	VOLUME	α1(I) / GAPDH	
tRNA	~0020-R1C1	111.4	4844		
Rat-1	~0020-R1C2	85645	72040	1.19	1.10
Rat-1 / BB2.6	~0020-R1C3	148279	407730	0.36	0.33
Rat1 / BB0.7	~0020-R1C4	87764	198905	0.44	0.40
1302	~0020-R1C5	29351	107966	0.27	0.10
1302 / BB2.6	~0020-R1C8	40784	280830	0.14	0.03

α1(I) GAPDH (rat, α1(I) / GAPDH

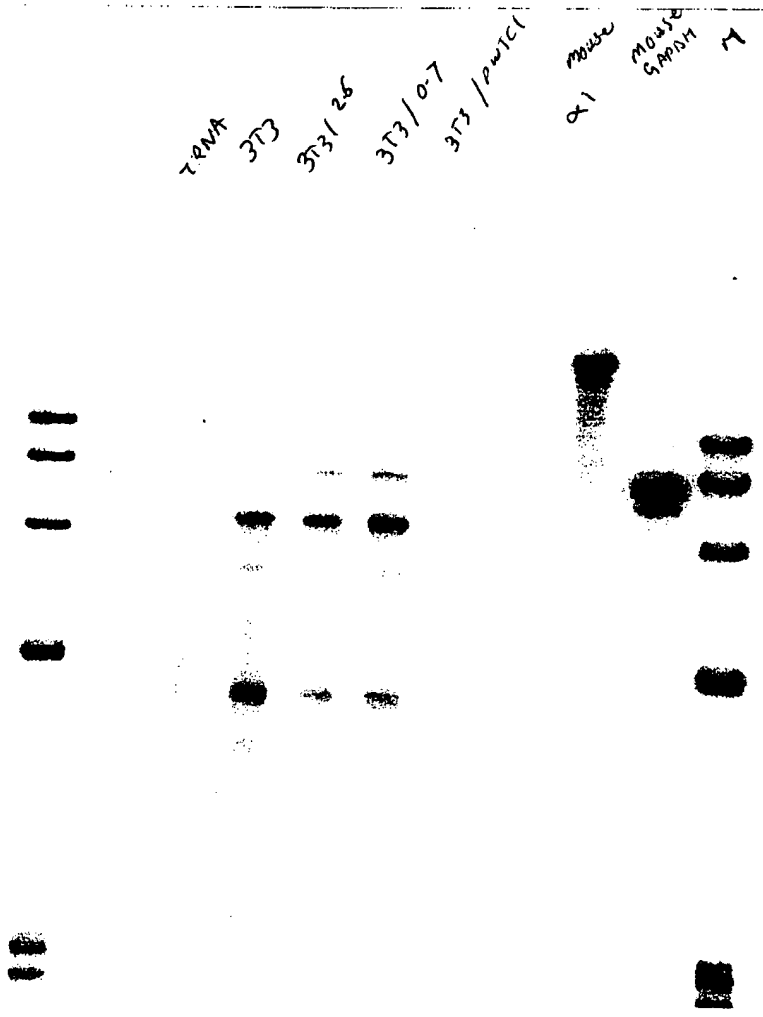
	OBJECT NAME		VOLUME	BACKGROUND	# PTS IN OBJECT	AVERAGE
αv1	3T3	01	26712	17.89	1581	34.88
	3T3/2.6	02	9628	15.28	969	25.22
	3T3/0.7	03	12067	16.73	1007	28.72
	3T3/pwTC1	04	1502	13.66	429	17.16
GAPDH	3T3	05	27211	18.63	1035	44.92
	3T3/2.6	06	26215	17.43	1225	38.83
	3T3/0.7	07	50919	18.99	1595	50.91
	3T3/pwTC1	08	5225	12.57	1025	17.66

3T3 0.98

3T3/2.6 0.36

3T3/0.7 0.24

3T3/pwTC1 0.29



DNA — treatment 1302 2.6 1302 0.7 1302 1302 2.6

373 373 2.6 373 0.7 373 pter

41 P Cannon 41/qtron DNA

Gebb

PCR

	µl	X 23	
10 X Buffer	10	230	7 µl/Sample
Primers Amp 3/5	4	92	
dNTP MIX (1 Smol, 1994)	1.4	32.2	
α^{32}P dCTP	1	23	
Taq Polymerase (Promega)	0.4	9.2	
H$_2$O	53.2	12 23.6	
DNA (1/10 of each DNA)	30 µl		

12 cycles

File #32, 33

1/10 of each DNA used in PCR

Amp Primers were used for all DNA quantitations

Thus:

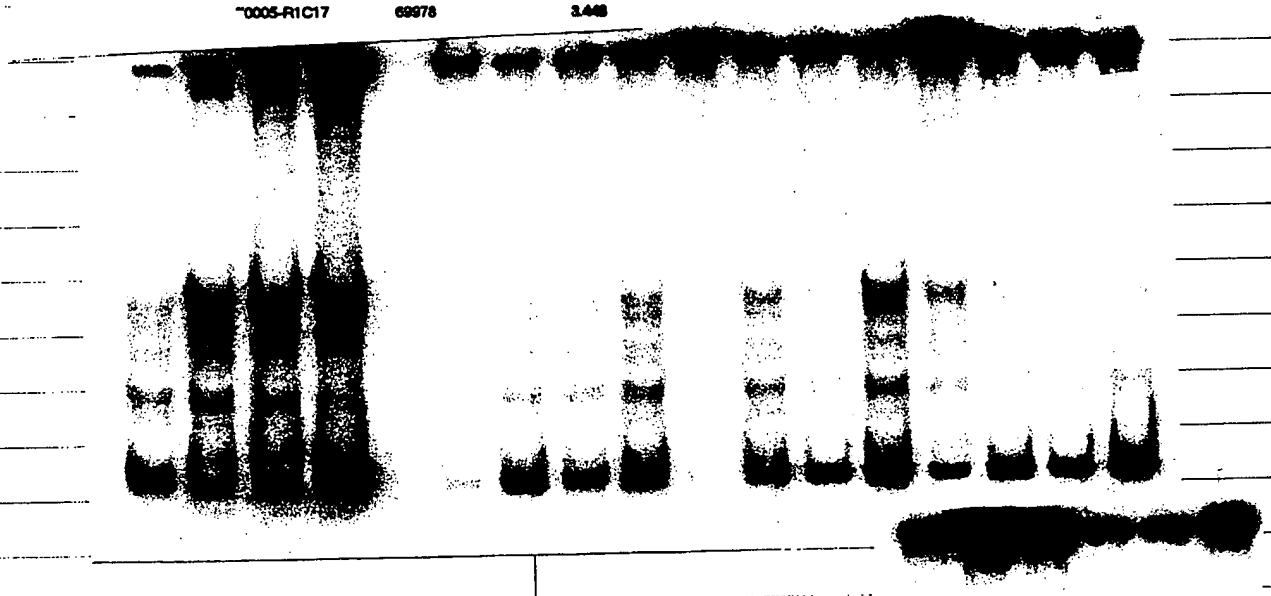
$$\text{Gene Copy No} = \text{ng read directly from the Standard curve} \times 2 \times 10^8 \times \frac{1}{\text{Fraction of DNA used in PCR}}$$

Total plasmid copy # $\times 10^7$

Rat-1 / 3.6 Kb promoter (Rat)	14
Rat-1 / 2.5 " " (mouse)	270
Rat-1 / 1.0 " " (Rat)	84
Rat-1 / 0.22 " " (mouse)	280
1302 / 3.6	56
1302 / 2.5	174
1302 / 1.0	76
1302 / 0.22	266
1-19 / 3.6	24
1-19 / 2.5	90
1-19 / 1.0	54
1-19 / 0.22	208
Rat-1 / BB 0.7	130
Rat-1 / BB 2.6	42
1302 / BB 2.6	52
3T3 / 0.7	18
3T3 / 2.6	28
3T3 / pWTC1	156

OBJECT NAME	VOLUME	BACKGROUND
"0005-R1C1	70562	4.129
"0005-R1C2	283159	5.476
"0005-R1C3	593639	6.532
"0005-R1C4	774686	6.225
"0005-R1C5	20334	4.334
"0005-R1C6	15121	3.981
"0005-R1C7	84978	4.060
"0005-R1C8	41412	3.984
"0005-R1C9	85320	4.081
"0005-R1C10	7861	3.953
"0005-R1C11	65157	4.076
"0005-R1C12	40515	3.799
"0005-R1C13	82279	3.966
"0005-R1C14	19363	3.888
"0005-R1C15	45047	3.885
"0005-R1C16	32617	3.781
"0005-R1C17	69978	3.448

OBJECT NAME	VOLUME	BACKGROUND
"0007-R1C1	14492	3.995
"0007-R1C2	85406	3.932
"0007-R1C3	42231	3.794
"0007-R1C4	87179	3.996
"0007-R1C5	8403	3.975
"0007-R1C6	66214	4.036
"0007-R1C7	39834	3.980
"0007-R1C8	85046	4.032
"0007-R1C9	20156	3.667
"0007-R1C10	44964	3.766
"0007-R1C11	32547	3.732
"0007-R1C12	72990	3.882



OBJECT NAME	VOLUME	BACKGROUND	# PTS IN OBJECT	AVERAGE
"0009-R1C1	54439	3.311	2544	24.71
"0009-R1C2	28585	3.497	2597	14.50
"0009-R1C3	32486	3.423	2597	15.93
"0009-R1C4	16897	3.519	2597	10.05
"0009-R1C5	51882	3.649	2597	12.84
"0009-R1C6	61289	3.578	2644	27.66